Biochemical and immunological characterization of multiple glycoforms of mouse mast cell protease 1: comparison with an isolated murine serosal mast cell protease (MMCP-4)

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Five highly soluble, chymotrypsin-like, neutral serine proteases, with molecular masses in the range 30–33 kDa, were isolated from *Trichinella spiralis*-infected mouse small intestine. These enzymes were closely related antigenically on Western blotting and by Ouchterlony double diffusion using a polyclonal, cross-absorbed, sheep antibody raised against mouse mast cell protease-1 (MMCP-1), and, on the basis of N-terminal amino acid sequence analysis, were identified as variant forms of MMCP-1. Substrate and inhibitor analysis confirmed that the five variants (MMCP-1 A–E) had similar characteristics, although highly significant ($P = 0.025$ to $P < 0.0001$) variations in K_m and $k_{cat.}$ were detected. Against human α_1-proteinase inhibitor the K_i for MMCP-1C (45 pM) was significantly ($P < 0.0001$) greater than those for the other proteases (0.76–2.2 pM). The differences in electrophoretic mobility are probably a result of variable glycosylation, since removal of N-linked carbohydrate produced a polypeptide of approx. 28 kDa in each case which was, like the native enzyme, immunoreactive on Western blotting. A much less soluble 28 kDa enzyme was isolated from serosal mast cells and identified as MMCP-4 by N-terminal amino acid sequencing. Like MMCP-1 it has chymotrypsin-like substrate specificities with activity at neutral pH. However, it was antigenically distinct from MMCP-1 and, using sheep anti-MMCP-1, was not detected on Western blotting or by Ouchterlony double diffusion, e.l.i.s.a. or immunohistochemistry. This last technique established that the MMCP-1 variants were uniquely present in enteric mast cells, thereby providing a highly selective means of distinguishing the mucosal and connective tissue mast cell subsets in the mouse.

INTRODUCTION

Neutral serine proteases have been identified as major secretory granule components in the mast cells of several species, including the mouse (Du Buske et al., 1984; Newlands et al., 1987), rat (Lagunoff and Pritzl, 1976; Woodbury et al., 1978), dog (Caughey et al., 1987; Vanderslice et al., 1990) and man (Schwartz et al., 1981; Schechter et al., 1986). The distinctive, apparently tissue-specific, distribution of mast cell proteases in man and rodents suggests that different mast cell populations may respond according to the type of protease secreted.

Rat connective tissue mast cells (CTMC), typified by those found at serosal surfaces, contain the chymotrypsin-like enzyme rat mast cell protease I (RMCP I) (Lagunoff and Pritzl, 1976), while mucosal mast cells (MMC), found predominantly in the mucosa of the gastrointestinal tract, contain the similar, but antigenically distinct and more soluble, enzyme, RMCP II (Woodbury et al., 1978). Quantification of the distribution and of the systemic secretion of RMCP II during intestinal immunological reactions have been facilitated by the development of highly sensitive e.l.i.s.a.s (reviewed in Miller et al., 1990), and it is clear from these studies that RMCP II serves a very different function from that of RMCP I.

Mouse mast cell protease (MMCP-1), isolated from the intestinal mucosa of mice infected with the parasite *Trichinella spiralis*, shares 74% amino acid sequence identity with RMCP II and also has a very similar tissue distribution and biochemical properties (Newlands et al., 1987; Miller et al., 1988; Le Trong et al., 1989; Huntley et al., 1990a). However, the protease content of murine mast cells is apparently more complex than that of the rat. Murine bone marrow-derived mast cells (BMMC), often regarded as *in vitro* analogues of MMC (Sredni et al., 1983), contain four mast cell proteases in the molecular mass range 28–33 kDa, including MMCP-1 as determined by Western blotting and e.l.i.s.a. (Newlands et al., 1991). Four esterases of similar or identical molecular masses which bind the specific serine protease inhibitor [^{3}H]di-isopropylfluorophosphate (DFP) have also been found in BMMC (Du Buske et al., 1984). Five proteases have been identified in CTMC and/or virus-immortalized mast cell lines on the basis of N-terminal amino acid sequences and molecular masses, and designated MMCP-2–MMCP-6 (Serafin et al., 1990; Reynolds et al., 1990). All but one (MMCP-3) of these proteases have been cloned and sequenced. MMCP-1 is expressed only in MMC and MMCP-2 is expressed in intestinal mucosa and Kirsten sarcoma virus-immortalized mast cells (KiSV-MC) (Serafin et al., 1991). MMCP-4 is transcribed in intestinal mucosa, CTMC and KiSV-MC (Serafin et al., 1991), and MMCP-5 in CTMC and KiSV-MC (Huang et al., 1991; McNeil et al., 1991). MMCP-6, identified as a tryptase by its predicted amino acid sequence, appears to be exclusive to CTMC and KiSV-MC (Reynolds et al., 1991).

The purpose of the present study was to define more fully the

Abbreviations used: α_1-PI, α_1-proteinase inhibitor; BMMC, bone marrow-derived mast cells; Cbz-L-Tyr-4NPE, carboxybenzoyl-L-tyrosine-4-nitrophenyl ester; CTMC, connective tissue mast cells; DFP, di-isopropylfluorophosphate; FITC, fluorescein isothiocyanate; HBSS, Hanks balanced salt solution; HRP, horseradish peroxidase; KiSV-MC, Kirsten sarcoma virus-immortalized mast cells; MMC, mucosal mast cells; MSMCP, mouse serosal mast cell protease; MMCP, mouse mast cell protease; PMSF, phenylmethanesulphonyl fluoride; RMCP, rat mast cell protease; Succ-Ala-Ala-Pro-Phe-4NA, succinyl-alanine-alanine-proline-phenylalanine-4-nitroanalide; t-PA, tissue plasminogen activator.

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MMCP-1-like proteases present in *Trichinella*-infected mouse intestine (Miller et al., 1990) and to compare their enzymic and antigenic properties with those of a novel, highly insoluble protease isolated from murine serosal mast cells.

MATERIALS AND METHODS

Male and female NIH mice, 8–10 weeks old, bred and maintained under conventional conditions at the Department of Zoology, University of Nottingham, U.K., were infected with 300 *T. spiralis* muscle larvae as described previously (Wakelin and Lloyd, 1976). Female Balb/c mice, 6 weeks old, were infected with 300 *Nippostrongylus brasiliensis* l_3 by subcutaneous injection. The mice were killed by cervical dislocation 10 days after infection. The small intestine of each mouse was removed and the lumen flushed through with Hanks balanced salt solution (HBSS) to remove digesta before storage at −20 °C or fixation in Carnoy's fluid and subsequent processing to paraffin wax for immunohistochemistry as described previously (Newlands et al., 1990). Porton mice, 6 months old, bred and reared at Moredun Research Institute, were used for isolation of murine serosal mast cell protease (MSMCP).

Isolation of enzymes

Murine intestinal mast cell proteases were isolated by a modification of the previously published method (Newlands et al., 1987). Briefly, after thawing, 10 g of small intestine was chopped finely with scissors and homogenized in 20 mM Tris/HCl, pH 7.5 (3 ml/g) with a Polytron homogenizer (Northern Media Supplies Ltd., North Cave, North Humberside, U.K.). The homogenate was centrifuged at 5000 *g* for 5 min and the resulting supernatant was centrifuged at 50000 *g* for 15 min. The final supernatant was applied to a 16 mm × 65 mm CM-Sepharose cation-exchange column (Pharmacia, Milton Keynes, Bucks., U.K.) equilibrated with 20 mM Tris/HCl, pH 7.5. Bound proteins were eluted with a 0–1.0 M NaCl gradient over 90 ml. Fractions (5 ml) were collected and screened for chymase activity against the synthetic substrate carboxybenzoyl-L-tyrosine 4-nitrophenyl ester (Cbz-L-Tyr-4NPE) (Sigma) as described previously (Knox et al., 1986). Those fractions which contained enzyme activity were pooled and applied to a 16 mm × 160 mm column of Sephadex G25 (Pharmacia) in order to rapidly desalt and exchange the buffer for 50 mM Mes (Sigma), pH 6.0. The desalted material was applied to a Mono-S cation-exchange column (Pharmacia) equilibrated with 50 mM Mes, pH 6.0, and eluted with a 50–150 mM NaCl gradient over 20 ml. Individual peaks were collected and those with activity against Cbz-L-Tyr-4NPE were diluted 5-fold with 50 mM Mes, pH 6.0, re-applied to Mono-S and again eluted with a 50–150 mM NaCl gradient.

Analysis of MSMCP

Mice were killed by cervical dislocation following anaesthesia with Halothane (May and Baker) and cells were recovered from the peritoneal cavity by lavage with 5 ml of HBSS containing 0.1% (w/v) gelatin (Hanks/gelatin). Cells were sedimented by centrifugation (400 *g* for 10 min), washed once with Hanks/gelatin and resuspended in 1 ml of Hanks/gelatin. Mast cell numbers were evaluated using a haemocytometer after staining with 1% (w/v) Methylene Blue in 50% (v/v) propylene glycol. The cell suspension was again sedimented by centrifugation; the supernatant fluid was discarded and the cell pellet was stored at −20 °C.

A mast cell granule preparation was prepared by the method of Lagunoff and Pritzl (1976). Briefly, a thawed cell pellet was resuspended in 1 ml of distilled water and the cells were fully disrupted by sonication (MSE Sonicator) at 16 μm amplitude for 3 × 10 s with a 3 mm diameter probe. Granules were isolated from the lysate by a differential centrifugation technique, first at 200 *g* to remove nuclei and larger cell debris followed by centrifugation at 50000 *g* for 15 min to sediment the mast cell granules. The granule pellet was washed twice with PBS and finally solubilized in 1 ml of 2 M NaCl. The pellet extract was further centrifuged at 8000 *g* for 2 min in an Eppendorf bench-top centrifuge and the supernatant fluid was applied to a Sephacryl S-200 (Pharmacia) size exclusion column (10 mm × 200 mm) equilibrated with 1.0 M NaCl in 20 mM Tris/HCl, pH 7.5. Fractions (1 ml) were collected and tested for enzyme activity against Cbz-L-Tyr-4NPE. Those containing activity were pooled, diluted to a final concentration of 0.5 M NaCl with 20 mM Tris/HCl (pH 7.5) and applied to the Mono-S cation-exchange column equilibrated with 0.5 M NaCl in 20 mM Tris/HCl, pH 7.5. The column was eluted with a 0.5–1.0 M NaCl gradient over 10 ml. All centrifugation and extraction steps were carried out at 4 °C.

Protein estimations

Protein concentrations were estimated using a Pierce BCA protein assay kit (Pierce and Warriner, Cheshire, U.K.), using BSA as standard, in accordance with the manufacturer's instructions.

SDS/PAGE analysis

Discontinuous SDS/PAGE was carried out as described by Laemmli (1970) on 15% mini slab gels (Mini Protein II; Bio-Rad) run at 200 V for 1.5 h, or on large format (Protean II xi; Bio-Rad) 10% tricene/SDS/PAGE gels (Schagger and von Jagow, 1987) run overnight at 100 V (constant) to prepare samples for amino acid sequence analysis. A range of molecular mass standards was included in each run: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), bovine carbonic anhydrase (30 kDa), ovalbumin (45 kDa), BSA (66.2 kDa) and phosphorylase B (97.4 kDa). Protein bands were visualized by staining with Coomassie Blue or by silver staining.

Electroblotting

Proteins in SDS/PAGE gels were transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) or poly(vinylidene difluoride) membranes (Immobilon P; Millipore) using a semi-dry transfer apparatus (Kyhse-Andersen, 1984) with a current of 0.1 mA/cm² of gel for 1 h. After transfer, nitrocellulose membranes were incubated in a solution of 0.1% (w/v) Tween 20 (Sigma) in PBS, pH 7.5 (PBS/T20), for 30 min at room temperature to block non-specific protein adsorption to the membranes. Following the blocking procedure the membranes were rinsed in fresh PBS/T20 (all subsequent washes and antibody dilutions were in PBS/T20) and transferred to optimally diluted sheep anti-MMCP-1, rabbit anti-MMCP-1 or rabbit anti-RMCP I for 1 h at room temperature. The membranes were subsequently washed (3 × 5 min) and probed with optimally diluted pig anti-sheep IgG–horseradish peroxidase (HRP) conjugate prepared according to the method of Nakane and Kawaoi (1974), or with sheep anti-rabbit IgG–HRP conjugate (Sera, W. Crawley, Sussex, U.K). Peroxidase activity was revealed with diaminobenzidine/H_2O_2 (Graham and Karnovsky, 1966). Immobilon membranes with protein bands for sequence analysis were stained with Coomassie Blue immediately after transfer was completed, and destained in methanol.

Deglycosylation

Aliquots of the proteases (20–25 μl), containing approx. 2 μg of protein, were denatured by heating in a boiling-water bath for 10 min and then cooled to room temperature. Denatured samples were incubated with 10 μl of deglycosylation buffer [150 mM phosphate buffer, pH 7.0, 50 mM EDTA and 1% (v/v) 2-mercaptoethanol] and 1 μl (0.2 unit) of peptide-N-glycosidase F or endo-α-acetylgalactosaminidase (Boehringer–Mannheim, Lewes, E. Sussex, U.K.) at 37 °C overnight. An additional 0.2 unit of glycosidase was then added and incubation was continued for a further 24 h.

E.l.i.s.a.

MMCPs were quantified by an antibody capture e.l.i.s.a. described previously (Huntley et al., 1990a) except that the capture antibody was affinity-purified sheep anti-MMCP-1, which was diluted to 1 μg/ml for coating e.l.i.s.a. plates.

Immunohistochemistry

Tissue sections were stained with Toluidine Blue, pH 0.5 (Enerback, 1966), or with sheep anti-MMCP-1 directly conjugated to fluorescein isothiocyanate (FITC) (Rinderknecht, 1962), by the following method. Rehydrated sections (5 μm thick) were incubated in 5% (w/v) BSA (Sigma, Grade IV) in PBS for 30 min before transfer to sheep anti-MMCP-1–FITC, optimally diluted in 5% BSA/PBS for 1 h at room temperature. After washing (3 × 5 min in PBS), sections were mounted in Citifluor non-fluorescent mountant (Citifluor Ltd., London, U.K.).

Specific anti-protease antibodies

Rabbit antiserum to MMCP-1 was prepared as described previously (Newlands et al., 1987). Rabbit anti-RMCP I antibodies were raised, absorbed against MMCP-1-Sepharose and affinity purified on RCMP I–Sepharose as described previously (Miller et al., 1988). Antiserum to MMCP-1 was raised in sheep by intramuscular injection of 100 μg of MMCP-1 in Freund's complete adjuvant. Two subsequent injections of MMCP-1 in Freund's incomplete adjuvant were given 4 and 7 weeks after the first. The sheep was bled at 2-week intervals after the final injection and batches of serum with an antibody titre >1:16 by double diffusion against MMCP-1 were applied to an MMCP-1–Sepharose affinity column and eluted anti-MMCP-1 antibodies were cross-absorbed against RMCP I as described for rabbit anti-MMCP 1 (Miller et al., 1988).

Binding of [^{3}H]DFP

Protease samples (40 μl) containing 4–8 μg of protein were incubated with 10 μCi of [^{3}H]DFP (5.8 Ci/mmol; Amersham International plc) at 37 °C for 15 min. The samples were prepared for electrophoresis by the addition of 50 μl of reducing sample buffer (Laemmli, 1970) and heating to >90 °C for 3 min. After electrophoresis the gels were prepared for autoradiography by the method of Laskey and Mills (1975). Radiographs were exposed at −70 °C and developed after 21 days.

Substrate kinetics

All kinetic measurements were made using a Beckman DU 600 spectrophotometer. Rates of hydrolysis of five concentrations (0.125–2 mM) succinyl-alanine-alanine-proline-phenylalanine-4-nitroanalide (Succ-Ala-Ala-Pro-Phe-4NA) were measured by adding 2 μl of substrate solution to 48 μl of enzyme solution in 0.1 M Tris/HCl, pH 7.5. The increase in absorbance was continuously measured at 410 nm, and the spectrophotometer's on-board software was used to calculate the kinetic constants K_m and $k_{cat.}$ from the initial rates of substrate hydrolysis.

Concentrations of protease were determined by active-site titration of the MMCP-1-like proteases against the fluorogenic substrate 4-methylumbelliferyl-*p*-(*NNN*-trimethylammonium)-cinnamate (Sigma) (Jameson et al., 1973). Fluorescence was measured on a Perkin–Elmer 3000 fluorescence spectrophotometer with an excitation wavelength of 365 nm and an emission wavelength of 445 nm. Unknown samples were measured, in triplicate, against a standard curve prepared with 7-hydroxy-4-methylcoumarin (Aldrich Chemical Co. Ltd.).

Inhibitor sensitivity

The sensitivity of isolated enzymes to the following specific protease inhibitors was studied using Cbz-L-Tyr-4NPE as substrate: 2.0 mM phenylmethanesulphonyl fluoride (PMSF), 20 μM 3,4-dichloroisocoumarin, 200 μM *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-amido-(4-guanido)butane (E64), 2 mM 1,10-phenanthroline and 1 mM pepstatin (all reagents supplied by Sigma). The assay of enzyme activity was as follows. Samples of 2.5 μl of enzyme preparation and 2.5 μl of inhibitor were preincubated with 40 μl of 0.1 M Tris/HCl buffer, pH 7.5, at 22 °C for 1 h. The enzyme concentration in the incubation mixture was 50 nM and the inhibitor concentrations were those given above. After preincubation, 5 μl of substrate was added and incubated for a further 3 min. Enzyme activity was quenched by the addition of 450 μl of 70% (v/v) methanol. Absorbance was measured at 410 nm and the percentage inhibition was calculated in comparison with an enzyme preparation incubated without inhibitor.

The association constant ($k_{ass.}$) for the reaction between the MMCP-1-like proteases and human α_1-proteinase inhibitor (α_1-PI; Sigma) was determined by preincubating equimolar enzyme and α_1-PI for periods of 15–120 s before continuous measurement of the rate of hydrolysis of the substrate Succ-Ala-Ala-Pro-Phe-4NA. The $k_{ass.}$ for each reaction was calculated by non-linear regression as described previously (Pirie-Shepherd et al., 1991). The inhibition constant (K_i) for the reaction between each protease and α_1-PI was determined by incubating the protease with various inhibitor concentrations (0.25–1.75 molar equivalents) for $35 \times t_{\frac{1}{2}}$ (the time to half-inhibition for an equimolar reaction, calculated from the equation $t_{\frac{1}{2}} = 1/k_{ass.}E_0$, where E_0 is the initial enzyme concentration). The apparent K_i ($K_i^{app.}$) was calculated by non-linear regression, as described previously (Pirie-Shepherd et al., 1991), and the true K_i was calculated from the equation $K_i = K_i^{app.}/(1 + S_0/K_m)$, where S_0 is the initial substrate concentration.

Amino acid analysis

N-terminal sequence analyses of MMCP-1 A–E were performed on soluble proteins in 50 mM Mes directly from ion exchange on an Applied Biosystems 477A protein microsequencer by the Welmet Protein Characterization Facility, Biochemistry Department, University of Edinburgh. MSMCP, immobilized on Immobilon-P following tricene/SDS/PAGE and Western blotting, was sequenced in the Microchemical Facility, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, U.K.

RESULTS

Enzyme isolation

Enteric proteases

Cation-exchange chromatography of small intestinal homogenate in 20 mM Tris/HCl buffer, pH 7.5, on CM-Sepharose, when eluted with a linear 0–1.0 M NaCl gradient, gave one major peak which contained all the detectable enzyme activity against the synthetic substrate Cbz-L-Tyr-4NPE. After buffer exchange into 50 mM Mes, pH 6.0, the enzyme activity was resolved into five distinct peaks by f.p.l.c. cation-exchange chromatography on Mono-S (Figure 1). Each peak containing enzyme activity was collected separately, and after 5-fold dilution it was re-chromatographed on Mono-S, where each eluted at a different molarity (88, 92, 103, 109 and 115 mM NaCl for peaks 1–5 respectively). These fractions were labelled MMCP-1A to -1E, in order of elution, for identification.

Analysis of the peaks by SDS/PAGE under reducing conditions (Figure 2a) showed MMCP-1A to contain two polypeptides of 32 and 33 kDa of different staining intensities, which were not resolved by the techniques used. MMCP-1B and -1C each contained single proteins of 31 kDa, and MMCP-1D and -1E each contained a single band of 30 kDa.

MSMCP

Size-exclusion chromatography of the serosal mast cell granule extract on Sephacryl S-200 resolved two major peaks, the second of which contained enzyme activity which hydrolysed Cbz-L-Tyr-4NPE. When this material was applied to Mono-S cation exchanger, a single peak of bound material was eluted at 0.77 M NaCl. The protein resolved by cation-exchange chromatography hydrolysed Cbz-L-Tyr-4NPE and contained a single 28 kDa polypeptide on SDS/PAGE (Figure 2a).

Biochemical characterization

The MMCP-1 polypeptides in the 30–33 kDa range all bound the group-specific inhibitor [^{3}H]DFP (Figure 3), indicating the presence of serine esterase activity. The autoradiograph of the labelled proteins showed the same pattern as the silver-stained SDS/PAGE gel (Figure 2a), except that MMCP-1D had an additional polypeptide of 27 kDa labelled with [^{3}H]DFP which was not detectable on the stained gel (Figure 3). This additional poly-

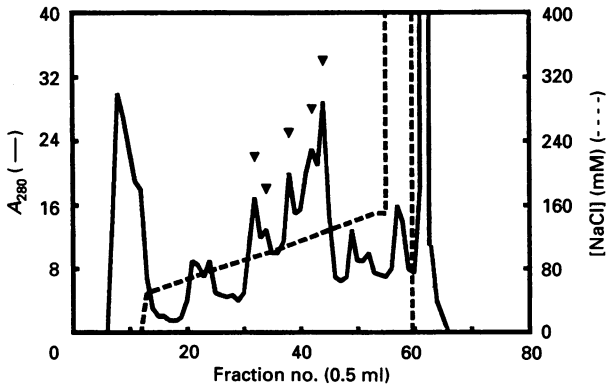

Figure 1 Mono-S cation-exchange chromatogram showing the resolution of five peaks of enzyme activity (arrowheads) against Cbz-L-Tyr-4NPE

The column was equilibrated with 50 mM Mes, pH 6.0, and eluted with a segmented gradient of 50–150 mM NaCl.

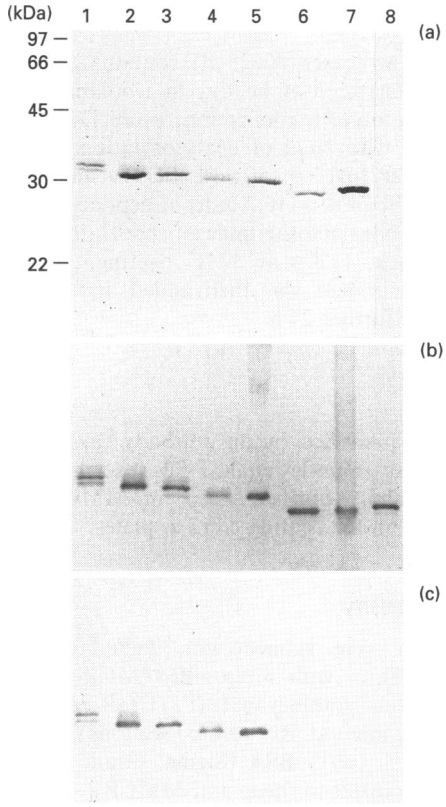

Figure 2 Analysis of MMCP-1 peaks

Characterization of MMCP-1 peaks from Mono-S cation-exchange on an SDS/15%-PAGE gel (silver staining). Peaks 1A–E from Mono-S were loaded in lanes 1–5 respectively. MSMCP is in lane 6, and RMCP I in lane 7. (**b**) and (**c**) Western blot analysis showing MMCP peaks 1A–E from Mono-S in lanes 1–5 respectively, MSMCP in lane 6, 10^4 purified mouse serosal mast cells in lane 7 and RMCP I in lane 8 probed with rabbit anti-MMCP-1 (**b**) or sheep anti-MMCP-1 absorbed to remove anti-RMCP I activity (**c**). Isolated enzymes were loaded at the rate of 40–60 ng per lane.

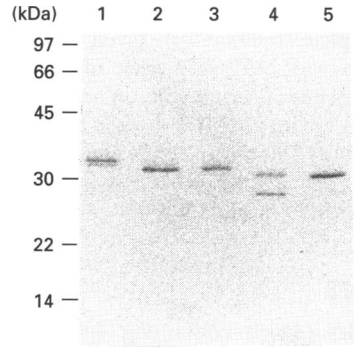

Figure 3 Autoradiograph of MMCP-1 peaks A–E labelled with the specific serine esterase inhibitor [^{3}H]DFP

Lanes 1–5 contain peaks A–E respectively.

Table 1 Substrate specificities of multiple forms of MMCP-1 and of MSMCP

Enzyme activity is expressed in nkat/mg of protein; 1 nkat is that activity which hydrolyses 1 nmol of substrate in 1 s. Bz, benzoyl; NA, no activity detected.

		Activity (nkat/mg)					
	Substrate	1A	1B	1C	1D	1E	MSMCP
Chymotryptic (esterase)	Cbz-L-Tyr-4NPE	63.0	42.0	31.0	33.0	21.0	259.0
	Cbz-L-Trp-4NPE	14.0	10.0	6.0	5.0	4.0	9.0
	Cbz-L-Phe-4NPE	0.3	0.9	NA	0.2	NA	NA
Elastinolytic (esterase)	Cbz-L-Ala-4NPE	15.0	6.0	4.0	9.0	4.0	41.0
	Cbz-D-Ala-4NPE	8.0	2.0	2.0	5.0	2.0	22.0
Typtic (esterase)	Cbz-L-Lys-4NPE	NA	NA	NA	NA	NA	NA
Chymotryptic (amide)	Bz-L-Tyr-4NA	1.0	NA	NA	NA	NA	2.0
	Succ-Phe-4NA	0.8	0.2	NA	NA	0.3	5.0
Tryptic (amide)	Cbz-L-Arg-4NA	2.0	NA	NA	0.4	NA	8.0
	Bz-DL-Arg-4NA	1.0	0.3	0.001	NA	NA	NA

Table 2 Substrate and inhibitor kinetics of the MMCP-1-like proteases

Conditions for substrate kinetics were 0.1 M Tris/HCl, pH 7.5, at 22 °C. Results were means ± S.E.M.

MMCP	K_m (μM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	$10^{-5} \times k_{ass.}$ ($M^{-1} \cdot s^{-1}$)	K_i (pM)
1A	922 ± 73.7	2.33 ± 0.10	2.5 ± 0.09	7.67 ± 1.50	0.76 ± 0.18
1B	991 ± 59.9	1.66 ± 0.04	1.7 ± 0.07	12.30 ± 0.35	1.02 ± 0.10
1C	663 ± 49.9	2.84 ± 0.12	4.3 ± 0.22	3.29 ± 0.48	45.40 ± 7.90
1D	936 ± 83.9	2.54 ± 0.12	2.7 ± 0.13	4.07 ± 0.62	2.20 ± 0.34
1E	832 ± 56.9	1.25 ± 0.04	1.5 ± 0.07	11.40 ± 0.22	1.51 ± 0.12
n	4	4	4	3	3
ANOVA	$P = 0.026$	$P < 0.0001$	$P < 0.0001$	$P < 0.001$	$P < 0.0001$

Table 3 Inhibition characteristics of mast cell proteases MMCP-1A–E and MSMC

Results are the percentage inhibition after 60 min of incubation with the inhibitor; $n = 3$ for each preparation.

	Inhibition (%)					
Inhibitor	1A	1B	1C	1D	1E	MSMC
PMSF	100	100	100	100	100	100
3,4-Dichloroisocoumarin	100	100	100	100	100	100
E64	5	18	5	9	0	0
1,10-Phenanthroline	8	5	11	5	0	0
Pepstatin	13	0	0	0	7	0

Table 4 MMCP concentrations and mast cell numbers in *N. brasiliensis*-infected and normal mice

Jejunal mast cell counts are expressed as cells per villus/crypt unit (vcu). *A, mast cells were abundant but were not counted.

	Control		Infected	
	Jejunum	Tongue	Jejunum	Tongue
MMCP (μg/g)	13 ± 3.6	0	183 ± 23.7	0.5 ± 0.12
Mast cell no. (Toluidine Blue)	1 ± 0.2	A*	7 ± 0.7	A*
Mast cell no. (fluorescence)	1 ± 0.3	0	6 ± 0.3	0

peptide may represent a degradation product generated during incubation with [^{3}H]DFP.

The five MMCP-1-like enzymes and MSMCP were screened against a range of low-molecular-mass synthetic protease substrates and were found to exhibit chymotrypsin-like esterase activity, as reported previously for MMCP-1 (Newlands et al., 1987); the results are summarized in Table 1. MSMCP also had chymotrypsin-like substrate specificities (Table 1). Using Succ-Ala-Ala-Pro-Phe-4NA as substrate, kinetic constants were calculated for each of the five MMCP-1-like enzymes. There were significant differences in K_m values ($P = 0.025$) with this substrate, and highly significant differences in both $k_{cat.}$ and $k_{cat.}/K_m$ ($P < 0.0001$ for each) by one-way analysis of variance. These results are summarized in Table 2.. The pH optima lay in the range pH 7.5–7.8 for all of the isolated enzymes (results not shown).

The five intestinal MMCPs and MSMCP were all completely inhibited by PMSF and 3,4-dichloroisocoumarin, both of which are inhibitors of serine proteases, but not by 1,10-phenanthroline, E64 or pepstatin, which inhibit metallo-, thiol and aspartic

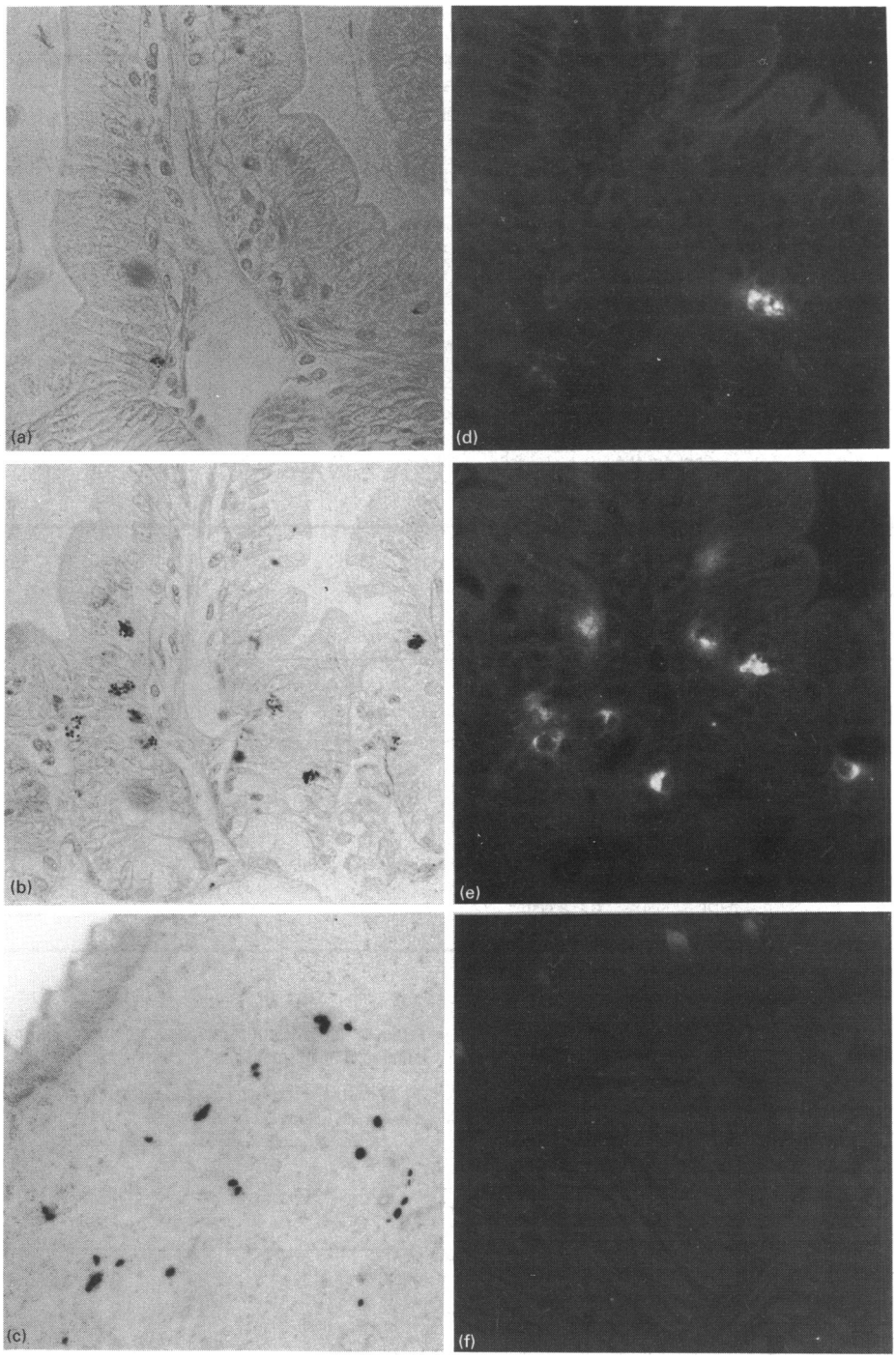

Figure 4 Photomicrographs showing normal mouse small intestine, *T. spiralis*-infected mouse small intestine and normal mouse tongue stained with Toluidine Blue, pH 0.5 (A–C respectively), or sheep anti-MMCP-1–FITC (D–F).

proteases respectively (Table 3). The MMCP-1-like proteases were also inhibited by human α_1-PI while MSMCP, like RMCP I in the rat (Pirie-Shepherd et al., 1991), was not. The association constants ($k_{ass.}$) for the reactions between the MMCP-1-like proteases and α_1-PI showed highly significant differences by one-way analysis of variance ($P = 0.001$). Inhibition constants obtained for the interaction of the MMCPs with α_1-PI showed K_is in the picomolar range, with the K_i of MMCP-1C being almost 60-fold greater than that of MMCP-1A ($P < 0.0001$; Table 2).

Immunological analysis

The isolated MMCPs were all identified by the rabbit antiserum raised against MMCP-1 (Newlands et al., 1987; Figure 2b) on Western blots, as was the 28 kDa MSMCP (Figure 2b). When a similar blot was probed with sheep anti-MMCP-1, cross-absorbed against RMCP I, only the highly soluble antigens isolated from small intestine were identified (Figure 2c).

The five MMCP-1-like enzymes shared complete lines of

	1	5	10	15	20	25	29
MMCP-1A	I	I G G V E A R P H S R P Y M A H L K I I T D R G S E D R					
MMCP-1B	I	I G G V E A R P H S R P Y M					
MMCP-1C	I	I G G V E A R P H S R P Y M A H L K I I T D R G S E D R					
MMCP-1D	I	I G G V E A R P H S R P Y M A H L K I I T D R G					
MMCP-1E	I	I G G V E A R P H S R P Y M A H L K I I T D R G					
MSMCP	I	I G G V E S R P H S R P Y M A H L E I					

Figure 5 N-terminal amino acid sequences of the variant glycoforms of MMCP-1 (A–E) and MSMCP (MMCP-4)

The single letter code is used to identify amino acids.

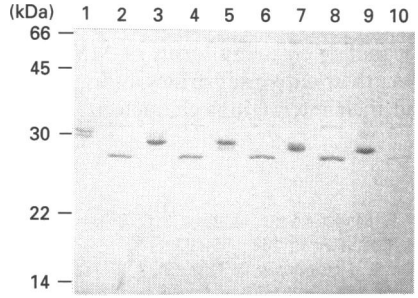

Figure 6 Silver-stained SDS/PAGE gel showing MMCP-1A to -1E before (lanes 1, 3, 5, 7 and 9 respectively) and after (lanes 2, 4, 6, 8 and 10 respectively) removal of N-linked carbohydrate with peptide-N-glycosidase F

identity in gel diffusion against sheep anti-MMCP-1, whereas no precipitation occurred with MSMCP (results not shown). When sheep anti-MMCP-1 was substituted with sheep anti-(RMCP I) a precipitin line was uniquely present against MSMCP and there was no reaction against MMCP-1 (results not shown).

All five MMCPs (0.5–12 ng/ml) were detected by e.l.i.s.a. using sheep anti-MMCP-1 as capture antibody, whereas MSMCP, RMCP I and RMCP II were not detected at 270 ng/ml, 2.5 μg/ml and 5.0 μg/ml respectively. After establishing the specificity of the e.l.i.s.a., samples of small intestine from *N. brasiliensis*-infected mice were assayed and MMCP-1 concentrations were found to be increased 14-fold when compared with uninfected control mice (Table 4). In contrast, tongues from control mice had no detectable MMCP-1 despite being rich in mast cells (Table 4; Figure 4). Small quantities of MMCP-1 were detected in tongues from infected mice (Table 4) and, as described previously (Huntley et al., 1990a), this is probably derived from the blood.

Immunohistochemical studies using the directly labelled sheep anti-MMCP-1–FITC conjugate confirmed the e.l.i.s.a. results, in that there were increased numbers of mast cells in the intestinal mucosa of parasitized mice which fluoresced after incubation with sheep anti-MMCP-1–FITC when compared with uninfected controls (Figure 4; Table 4). There were no mast cells detected by immunofluorescence in tongues of either control or parasitized mice despite the presence of abundant mast cells which stained with Toluidine Blue (Figure 4).

N-terminal sequencing

To determine whether the five intestinal mast cell proteases were separate gene products or differed because of post-translational processing, the N-terminal amino acid sequence was determined for each protease (Figure 5). The first 15–29 N-terminal residues of the intestinal mast cell proteases proved to be identical in each case and were in agreement with the previously published sequence for mouse intestinal mast cell protease (Le Trong et al., 1989). The amino acid sequence determined for MSMCP showed complete identity over the first 20 residues with that published for MMCP-4 (Reynolds et al., 1990).

Deglycosylation

To further investigate whether the differences between MMCP-1A–E are post-translational and a consequence of differential glycosylation, samples of each enzyme preparation were incubated with endo-α-acetylgalactosaminidase to remove O-linked carbohydrate, or with peptide-N-glycosidase F to remove N-linked carbohydrates. No change in the apparent molecular mass of any of the MMCPs, as determined by SDS/PAGE, was detected even after prolonged treatment with O-glycosidase. Following treatment of the five MMCPs with peptide-N-glycosidase F, however, there was degradation of each MMCP resulting in the appearance of a new polypeptide with an apparent molecular mass of 28 kDa (Figure 6). This 28 kDa polypeptide remained strongly antigenic on Western blot when probed with sheep anti-MMCP-1 (results not shown).

DISCUSSION

The five proteases isolated from *T. spiralis*-infected mouse small intestine can be identified as MMCP-1 by several criteria. Like MMCP-1, they are highly soluble, chymotrypsin-like proteases active at neutral pH. They are closely related antigenically both on Western blots and by Ouchterlony double diffusion, and are readily distinguished from MSMCP on the basis of their antigenicity. Finally, they share complete amino acid sequence identity over the first 15–29 N-terminal residues (Le Trong et al., 1989) which further distinguishes the MMCP 1 group of proteases from MMCPs 2–6 (Serafin et al., 1990; Reynolds et al., 1990), all of which differ from MMCP-1 within the first 10 N-terminal residues.

The differences in electrophoretic mobility between these variant forms of MMCP-1 are probably a result of variable glycosylation, since removal of N-linked carbohydrate moieties produced a new polypeptide of approx. 28 kDa from each of MMCP-1A–E which was antigenically similar to native MMCP-1. Analysis of the carbohydrate content of each enzyme preparation might prove helpful in further distinguishing between the different glycoforms of MMCP-1. It may be that each is produced by mast cells in different regions of the intestine or, more likely, by mast cells at varying stages of maturation, since intestinal nematodiasis induces massive mucosal mastocytosis with extensive recruitment and differentiation of intestinal mast cells (Miller et al., 1989).

Variable glycosylation occurs in other serine proteases, notably in the subunits of human mast cell tryptase (Cromlish et al., 1987) and tissue plasminogen activator (t-PA) (Wittwer et al., 1989). Human t-PA is synthesized in two forms, I and II, which share N-terminal amino acid sequences but have disparate carbohydrate side groups, the composition of which appears to be dependent on the cell line from which the t-PAs are derived (Wittwer et al., 1989). In the presence of a fibrinogen fragment stimulator, t-PA II will catabolize a synthetic substrate up to five times faster than does t-PA I (Wittwer et al., 1989), while type I is more resistant to cleavage by plasmin (Wittwer and Howard, 1990).

The kinetic data presented here show that there are significant differences in the rates at which the MMCP-1 proteases catalyse a low-molecular-mass substrate. This may be due to steric hindrance of substrate binding by the carbohydrate and suggests that glycosylation may be important in the catalysis of macromolecular protein substrates, possibly in aiding recognition of specific cleavage sites. The influence of glycosylation on the catalytic properties of MMCP-1 will not be fully understood until the native substrates are fully characterized.

The inhibition studies of the various glycoforms of MMCP-1 with human α_1-PI also clearly show that the catalytic properties of these enzymes are modified through their carbohydrate moieties, with highly significant variations in how rapidly they associate with inhibitor and how tightly they are bound. This may be important in prolonging enzyme activity in situations where immediate hypersensitivity reactions have increased vascular permeability, bathing surrounding tissues in serum containing high concentrations of serine protease inhibitors.

MSMCP, isolated from peritoneal CTMC, is, as far as we are aware, the first murine CTMC protease to be isolated in its native form and characterized biochemically. MSMCP is a neutral 28 kDa serine protease with chymotrypsin-like substrate specificities, properties it has in common with MMCP-1. It differs from MMCP-1 in that it is highly insoluble and, on cation-exchange chromatography, behaves like RMCP I which has a net charge of +18 (Le Trong et al., 1987). In contrast, MMCP-1 has similar chromatographic properties to RMCP II and has a net charge of +3 at neutral pH (Le Trong et al., 1989). N-terminal amino acid sequence analysis of MSMCP shows complete identity over the first 20 residues with the CTMC protease MMCP-4 (Reynolds et al., 1990; Serafin et al., 1991), differing at residues 7 and 19 from MMCP-1 A, C, D and E (MMCP-1B was not sequenced beyond residue 15). Based on this evidence, we can identify MSMCP as MMCP-4.

Immunological techniques have proved to be particularly useful in the analysis of protease distribution and function. For example, Woodbury et al. (1978) showed that RMCP I and II were readily distinguished by gel diffusion although they share 74% amino acid sequence identity. However, it was not possible to determine the cellular distribution of RMCP I and II without first preparing monospecific polyclonal antibodies by cross-absorption (Gibson and Miller, 1986) or by raising a monoclonal antibody against RMCP II (Huntley et al., 1990b). The distinction between the MMCP-1 family and MMCP-4 was, however, readily achieved with polyclonal sheep anti-MMCP-1 cross-absorbed against RMCP I and affinity purified on MMCP-1–Sepharose 4B. This was observed with gel diffusion, Western blotting, e.l.i.s.a. and immunohistochemistry. It was thus possible, using this antibody for immunohistochemistry, to confirm e.l.i.s.a. results (Huntley et al., 1990a) showing that the MMCP-1 family of proteases is not present in CTMC. As yet no antibodies are available which allow distinction between MMCP-1A–E, so it is not clear whether the different glycoforms are present in distinct regions of the small intestine or are being produced by MMC of varying maturity.

Using cDNA probes and Northern blotting, Serafin et al. (1991) detected expression of MMCP-4 in the intestines of parasitized mice. This may be in agreement with our findings of an RMCP I-like antigen expressed in mast cells in the gastrointestinal mucosae of *T. spiralis*-infected mice (Miller et al., 1988) although, in the rat, RMCP I is not present in intestinal mucosal mast cells (Huntley et al., 1990b). Serafin et al. (1991) were, however, unable to detect MMCP-4 in bone marrow-derived cultured mast cells by Northern blotting, although transcription of MMCP-4 has recently been reported in BMMC grown in medium containing the c-kit ligand growth factor (Gurish et al., 1992). An RMCP I-like antigen in murine BMMC, detected with anti-(RMCP I) by immunofluorescence and by immunoblotting of cell lysates, has a very similar or identical electrophoretic mobility to the mouse CTMC protease now identified as MMCP-4 (Newlands et al., 1991). It is therefore probable that MMCP-4 is present in BMMC grown in T-cell-conditioned medium, and the failure to detect it by Northern blotting may simply be due to the low levels of transcription. For example, murine BMMC contain < 120 ng of MMCP-1/10^6 cells (Newlands et al., 1991), compared with up to 34 μg of RMCP II/10^6 rat BMMC (Haig et al., 1988). These results again emphasize the potential value of highly specific antibodies in the analysis of protease distribution.

In summary, the five proteases isolated from mouse small intestine are identified as variant glycoforms of MMCP-1, and the novel CTMC protease is identified as MMCP-4. The biological significance of so many forms of MMCP-1 will only become clear when their native substrates and specific inhibitors are identified and their interactions characterized.

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